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THE ANTICOMPLEMENTARY ACTIVITY OF 'FUSOBACTERIUM POLYMORPHUM' I--ETC(U)
JAN 77 C E HAWLEY, W A FALKLER

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The Anticomplementary Activity of *Fusobacterium polymorphum* in Normal and C-4 Deficient Sources of Guinea Pig Complement.

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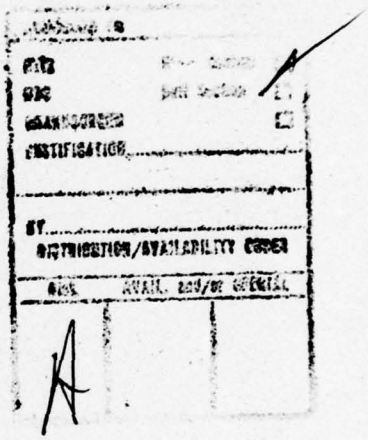

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Abstract

Fusobacterium polymorphum has been isolated from the gingival crevice in man, and has been implicated in the immunopathology of periodontal diseases. The presence of alternate complement pathway factors in gingival crevice material suggests the contribution of this process in the manifestation of the local host response. A complement consumption assay was used to show that the anticomplementary activity of a cell wall preparation from *F. polymorphum* in guinea pig complement progressed optimally at 37 C and suboptimally at 0 C. Similar levels of complement consumption were recorded at both temperatures, but a higher concentration of cell walls was required in order to demonstrate maximum activity in the cold. Augmentation experiments using additional normal and heat treated complement showed the presence of heat labile factors which controlled and limited the consumption of complement to a constant proportion of the total amount present. These same experiments indicated that there were no reactive antibodies in the complement used in the assay. Similar tests with C'4-deficient guinea pig sera confirmed that *F. polymorphum* cell walls were capable of generating alternate complement pathway activity in guinea pig sera.

Introduction:

Gram negative filamentous bacteria belonging to the genus *Fusobacterium* tend to increase numerically in dental plaque with time (21, 22, 23). In addition, recent reports indicate that *F. polymorphum*, also designated *F. nucleatum* (16), has been determined as one of the major anaerobic isolates from advanced cases of periodontitis (31, 36, and A. A. Crawford, S. S. Socransky, and G. Bratthall, 1975. J. Dent. Res. 54A: 97.). The finding of serum antibodies reactive with oral gram negative anaerobic organisms has implicated these indigenous crevicular microorganisms in the immunopathology of periodontal diseases (6, 12, 15, 18, 19, 26). This paper is a continuation of our investigations into the involvement of *F. polymorphum* as a potential etiologic agent in human periodontal disease.

It has been suggested that the histopathology of periodontal diseases is mediated through the participation of immune recognition and effector systems (17). One of the effectors is the complement system which is classically activated by the combination of immune complexes and the early acting complement components (9, 28). The products of classical pathway activation have shown phlogistic properties such as chemotactic, anaphylatoxic, and opsonic activity (4). The complement system may also be activated by mechanisms not involving specific immunoglobulins or the early complement components. An alternative activation pathway appears possible which involves the reaction of certain polymeric initiators (inulin, endotoxin, or zymosan) with non-immunoglobulin serum factors (13, 14, 29, 30, 32, 34, 35, 40). The subsequent cleavage of C'3 and the generation of biologically active complement fragments may also be significant to our understanding of the host response mechanisms in inflammatory disease states.

Bladen *et al.* have demonstrated the appearance of lytic lesions on the surface of *Veillonella alkalescens* when the organisms were grown in the presence of guinea pig (GP) sera. Similar lesions were produced in lipopolysaccharide

(LPS) particles that were exposed to GP sera. It was determined that the consumption of complement occurred primarily between C'3 and C'9, and that the reaction was characteristically inefficient at 0 C and optimally efficient at 37 C (3). Gewurz *et al.* also established that LPS, zymosan, and flagellin produced a greater than 80% reduction of total complement activity in GP sera (11). They reported that the anticomplementary activity of LPS on GP sera was directed toward the terminal components, C'3-C'9, and suggested that the activation of C'1, C'2, and C'4 may have occurred, but changes in hemolytic levels of these components could not be detected outside the sensitivity limits of the hemolytic assay (10).

Marcus *et al.*(29) attempted to resolve the issue of early component participation in the anticomplementary activity of LPS. LPS was incubated with GP sera prior to placing it in a complement consumption assay. The resulting complex of LPS and serum factors, LPS-X, displayed the ability to consume C'3, and anti-C'2 did not interfere with this activity. These findings suggested that there was an alternate (non-classical) pathway for the consumption of GP complement by LPS. A report by Phillips *et al.* (33) indicated that LPS, when bound to the surface of erythrocytes as E-LPS, would activate the complement cascade *via* reactions that involved antibody and the early acting complement components. It was also shown that the γ_2 globulin fraction of GP sera as well as small amounts of C'1, C'2, and C'4 were required for the expression of anticomplementary activity by E-LPS in GP sera. Later, it was revealed by Mergenhagen *et al.* (27) that different preparations of LPS had demonstrated the capability of initiating complement consumption by either the classical or alternate pathways.

With the discovery of a strain of guinea pigs with a genetic deficiency in C'4 (C4D), it was believed that sera taken from these animals could be used to

resolve the proposed alternate pathway activity by LPS (5). Frank *et al.* (7) were able to demonstrate that late acting complement components could be fixed by LPS in C4D sera. C'1 and C'2 were not consumed. In the same sera, immune complexes were shown to fix C'1 and C'2, but they were unable to effectively reduce C'3 through C'9 activity in the same sera. Sera from C4D guinea pigs have been effectively utilized to examine the activation of the alternate pathway by pneumococcal polysaccharides. The production of opsonically active C'3b was considered important to the host response against pneumococci (40).

Reports in the literature suggest that the ingredients (LPS and complement factors) for alternate pathway activation of the complement system are present in the diseased gingival crevice (2, 37, 38, 39). There is also evidence that alternate pathway activation has occurred in inflamed periodontal tissues (1). We present evidence here that the cell walls obtained from the gram negative anaerobe *F. polymorphum*, a major isolate of the diseased gingival crevice, is capable of activating the alternate complement pathway.

Materials and Methods

Growth of Microorganisms:

Fusobacterium polymorphum, ATCC #10953, were grown under anaerobic conditions using the BBL anaerobe jar-gas pak system. A liquid modified tryptone media was used which contained Bacto-tryptone (Difco), 10 g; Bacto-yeast extract (Difco), 10 g; K_2HPO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 1.25 g; glucose, 2 g; and sodium thioglycollate (BBL), 5 g per liter of distilled water. The pH of the media was 7.2 prior to autoclaving at 121 C at 15 pounds per square inch pressure for 20 min. After inoculation, the cultures were placed into the anaerobic growth system and incubated for 48 hr at 37 C.

Preparation of Cell Walls:

After incubation, the cells were harvested by centrifugation at 10,000 x g for 10 min at 4 C. The cells were then washed three times with 0.01 M phosphate buffered saline (0.15 M NaCl) pH 7.2 (PBS). The washed cells were then resuspended in isotonic saline and sonicated in a dry ice alcohol bath using a Branson sonicator with probe tip at 6 amps and 8 bursts of 30 sec each. The cell wall preparation of *F. polymorphum* was derived from the sonicated cellular debris according to the modified methods of Garcia *et al.* (8). Briefly, the sonicate was centrifuged at 2,000 x g for 10 min at 4 C. The sediment was resuspended in distilled water and then washed five times in distilled water at 20,000 x g for 15 min until the wash supernatants showed no absorbance for deoxyribonucleic acid at 260 nm. The cell wall preparation was lyophilized and the dry weight determined. Prior to their use in the complement consumption assay, the cell walls were reconstituted to a concentration of 5,000 µg/ml in deionized and distilled water. The preparation was designated FP cell walls.

Complement Titration:

Complement titration was performed according to a modified version of the LBCF method (20). Modifications included using 5 g dextrose per liter veronal buffered diluent (VBD). VBD prepared with the stock buffer containing Mg^{++} and Ca^{++} was designated VBD^{++}. A similar 5x stock buffer solution was also prepared which contained 42.5 g NaCl and no stock divalent cation solution, this was designated VBD^{--}.

Complement Consumption Assay in Normal GPC':

The methods of Bladen *et al.* (3), Gewurz *et al.* (10), and Philips *et al.* (33) were employed as the basis for the complement consumption assay. The

test consisted of placing 0.1 ml GPC', 0.1 ml of the experimental cell wall preparation (500 µg) or VBD as the negative control, and 0.8 ml VBD into a 12 x 75 mm serologic test tube. In addition, inulin was used in the assay at a final concentration of 500 µg/ml in place of the experimental cell walls as a positive control for complement consumption. The system was incubated in a water bath for 1 h at 37 C, or incubated in an ice bath for 1 h to 18 h at 0 C. Variations in the basic assay with GPC' included testing the effect of from 100 to 1,000 µg of the cell walls on a fixed amount of GPC' and testing the effect of 2 fold and 4 fold increases in normal and heat treated (56 C for 30 min) GPC' with a standard amount of cell walls.

At the end of the incubation period, the serologic tubes containing the assay mixtures were placed in an ice bath. A complement titration was performed on dilutions of the VBD controls. The number of $C'H_{50}$ units in the original 1 ml sample of undiluted GPC' was determined. From this value, the number of $C'H_{50}$ units contained in 0.3 ml of the assay mixtures could be calculated. Subsequently, 0.3 ml of the diluted and undiluted tests were added to 0.5 ml VBD. Then, 0.2 ml of the 3% suspension of EA was added, and the mixture was incubated in a water bath at 37 C for 30 min. The degree of hemolysis seen in the control and experimental tests was evaluated by single point analysis of partial lysis to determine residual complement activity. Conversion factors calculated from the Von Krogh Equation were employed in these computations (25). The results of the complement consumption assay of GPC' were expressed as total $C'H_{50}$ units consumed and also as a percent of the total complement found by titration in VBD controls.

Complement Consumption Assay of C-4 Deficient Guinea Pig Sera:

The complement consumption assay described above was modified to test

the reduction of total hemolytic complement activity in C-4 Deficient GP sera (C4D). The C4D was kindly provided by Dr. Jerry A. Winkelstein, Department of Pediatrics, The Johns Hopkins School of Medicine, Baltimore, Md., and was taken from descendants of those animals determined to have a genetically based deficiency in C'4 (5, 7, 40).

EAC'1,4 were prepared according to the methods suggested by Mayer (25). Briefly, SRBC were washed three times in VBD^{--} that had been supplemented by the addition of $CaCl_2$ at a final concentration of 0.001 M. A 3.0% suspension of these cells was made in the same buffer and sensitized with hemolysin (20). The EA were washed once with the $CaCl_2$ supplemented buffer, and a 3.0% suspension was prepared. To 7.0 ml of this suspension of EA, 0.35 ml reconstituted GPC' was added and allowed to react for 30 min at 0 C. The cells were then washed twice with the VBD^{--} supplemented with $CaCl_2$, resuspended in 14 ml of the same buffer, incubated for 90 min at 37 C, and sedimented by centrifugation in the cold. The resulting EAC'1,4 were resuspended in VBD^{--} and stored at 0 C until their use in the complement consumption assay with C4D. Prior to their use in the test, the EAC'1,4 were washed once in VBD^{++}, and 50 µl of a 3.0% suspension of the cells were tested with an equal volume of either C4D, GPC', or VBD^{++} to determine the potential ability of these cells to lyse under test conditions.

In the complement consumption assay system, 0.1 ml C4D was placed in a 12 x 75 mm serologic tube with 0.1 ml of either the cell wall preparation, inulin, or VBD^{++}. After the addition of an additional 0.8 ml VBD^{++}, the test mixture was incubated in a water bath for 1 h at 37 C. At the end of the incubation period, the reaction mixture was placed in an ice bath, and a complement titration was performed on the VBD^{++} controls using the EAC'1,4 cells. The experimentals, the inulin controls, and the VBD^{++} controls were evaluated for

residual complement activity using the EAC'1,4. Single point analysis of partial lysis was used to establish the degree of hemolysis, and the Von Krogh conversion factors were employed to compute $C'H_{50}$ unit activity (25). In addition, the same test solutions were examined with EA as a control for classical pathway in C4D. The results of the complement consumption assay with C4D were expressed as a percent of the total $C'H_{50}$ units found in VBD^{++} controls.

Results

Complement Consumption Assay of Normal Guinea Pig Complement:

An investigation was undertaken to evaluate the potential anticomplementary activity associated with the cell wall fraction of *F. polymorphum*. This activity was compared with that of other agents using test systems that were designed to test the effect of time, temperature, and concentration on the loss of hemolytic complement activity. The term "consumption" or "consumed" will be used in this paper to designate the loss of $C'H_{50}$ unit activity or the inability to titrate a predetermined level of complement activity by single point analysis of partial lysis. The standard from which all "consumption" was measured was the number of $C'H_{50}$ units in VBD controls as determined by complement titration.

F. polymorphum cell walls at dry weights of 31.25 µg, 62.5 µg, 125 µg, 250 µg, 500 µg, and 1,000 µg were tested in the complement consumption assay with 28.14 $C'H_{50}$ units of GPC' at 37 C for 1 h. The results shown in Figure 1 indicate that 31.25 µg and 62.5 µg of the cell walls showed minimal consumption of complement activity. Increasing the concentration to 125 µg produced a sharp elevation in the number and percent of C' units consumed. The increase through 250 µg and 500 µg was more gradual with no apparent increase in anti-

complementary activity of the cell walls at 1,000 µg in the test. These results suggested that at a concentration of 500 µg the optimum anticomplementary activity of the cell wall preparation had been reached.

A similar titration was performed using the same concentrations of cell walls and an additional test containing 2,000 µg. In this instance, the temperature of incubation was maintained in the cold at 0 C for 1 h. The results are shown graphically in Figure 2 and indicate that there was a gradual increase in complement consumption with increases in concentration of cell walls in the assay. The lower incubation temperatures produced consumption of 12.2% and 20.2% for 500 µg and 1,000 µg respectively. A comparison with the percent consumption attained by the same concentrations at 37 C reveals that the plateau levels of activity seen at 37 C could not be demonstrated at 0 C. Increasing the concentration of cell walls to 2,000 µg produced complement consumption to a level of 60.1%.

The complement consumption assay was used to determine the heat lability of factors in GPC' which might augment the reaction of a sub-plateau concentration of the cell walls. For this purpose, 125 µg of the antigen was chosen since it had been shown in earlier tests to reduce the hemolytic complement levels by only 39.7% compared to a maximum of greater than 50% by 500 µg. The basic test contained 33.33 titratable $C'H_{50}$ units of GPC'. The test was augmented with either 33.33 or 99.99 additional $C'H_{50}$ units of normal GPC'. A parallel test that used 2 fold or 4 fold increases of heat treated (56 C for 30 min) GPC' was also performed. The results of tests incubated for 1 h at 37 C shown in Table [1] indicate that increasing $C'H_{50}$ units of GPC' were consumed by the standard of 125 µg of cell walls as more normal GPC' was put into the test. With a two fold increase in units present, there was an approximate two fold increase (14.64 to 27.06) in units consumed. Similarly, with a four fold increase in

units present, there was a slightly greater than four fold increase (14.64 to 68.28) in consumption. With the addition of normal GPC', it was observed that the percent consumption of $C'H_{50}$ units remained essentially the same. In the tests where the heat treated GPC' was added there was no detectable increase in complement unit consumption. Controls of the basic test included the reaction of 500 µg inulin and 500 µg FP cell walls with the 33.33 $C'H_{50}$ units.

Table 1. CONSUMPTION OF GPC' BY CELL WALLS WHEN SUPPLEMENTED BY NORMAL AND HEAT TREATED GPC'

Amount $C'H_{50}$ units in test	Units consumed / Total units present in Test	Percent Consumption	Additional units Consumed
33.33	14.64 / 33.33	43.9%	----
66.66	27.06 / 66.66	40.6%	12.42
133.32	68.28 / 133.32	51.2%	53.64
66.66'	14.64 / 33.33	43.9%	-0-
133.32"	14.64 / 33.33	43.9%	-0-
33.33[a]	18.33 / 33.33	55.0%	----
33.33[b]	20.00 / 33.33	60.2%	----
VBD Control	0.63 / 33.33	1.9%	

'-33.33 of the 66.66 units present had been heat treated prior to use in the complement consumption assay
"-99.99 of the 133.32 units present had been heat treated prior to use in the complement consumption assay
[a]-control with 500 µg cell walls
[b]-control with 500 µg inulin

Complement Consumption Assay of C4-Deficient Guinea Pig Sera (C4D).

Prior to their use in the titration for residual complement activity in C4D, EAC'1,4 cells at a 3% suspension in VBD^{++} were tested for their ability to lyse in the presence of GPC' and C4D. A microtiter test was used in which 50 µl of the 3% EAC'1,4 cell suspension was added to 50 µl of both sources of complement or VBD^{++}. A 3% suspension of EA was tested in the same manner. All EA cells lysed in 20 min at room temperature in the presence of

normal GPC', however, EA did not lyse in C4D or VBD^{++} and formed a button in 1 h. In contrast, all EAC'1,4 cells lysed in both normal GPC' and C4D in 20 min at room temperature. In VBD^{++}, the same EAC'1,4 cells settled to a button in 1 h.

The results of the complement consumption in normal GPC' and C4D are shown in Table 2. Both *F. polymorphum* cell walls and inulin at a concentration of 500 µg in the test were capable of reducing the complement activity in normal GPC' and C4D. The cell walls consumed complement activity by 44.5% in normal GPC' and 32.4% in C4D. Inulin also consumed complement activity from both sera at a rate of 48.7% from normal GPC' and 22.3% from C4D. In addition, diluted samples of the C4D negative controls were titrated with EA cells. This test revealed that the ability of EA cells to lyse in normal GPC' was inhibited by more than 90% in C4D.

Table 2. CONSUMPTION OF C'H_{50} UNITS IN C'4-DEFICIENT GP SERA

Anticomplementary agent/complement source	Units consumed / Total units present in test	Percent consumption
Cell Wall/GPC'	8.34 / 18.15	44.5%
Cell Wall/C4D	10.17 / 31.35	32.4%
Inulin/GPC'	9.14 / 18.75	48.7%
Inulin/C4D	6.99 / 31.35	22.3%
EA/GPC'/VBD control	1.25 / 18.15	6.7%
EAC1,4/C4D/VBD control	2.29 / 31.35	7.3%
Inhibition of EA Classical Pathway EA/C4D		>90%

A test using C4D sera was performed to determine if increased consumption could be recorded by increasing the amount of cell walls in the test. The tests were incubated for 1 h at 37 C. The results of the tests are shown in Table 3. Complement was consumed with increasing concentrations of cell walls in the test.

Table 3. CONSUMPTION OF $C'H_{50}$ UNITS IN C4-DEFICIENT GP SERA

Anticomplementary agent	Units consumed / Total units present in Test	Percent consumption
FP Cell Wall (125)	19.34 / 51.93	37.2%
FP Cell Wall (250)	25.76 / 51.93	48.6%
FP Cell Wall (1,000)	37.36 / 51.93	73.1%
EAC1,4/C4D/VBD Control	0.99 / 51.93	1.9%

Discussion

In the complement consumption assay, the standard from which all consumption was measured was the number of $C'H_{50}$ units in VBD controls as determined by complement titration. In order to preclude the need for the laborious titration of the numerous tests of complement consumption by *F. polymorphum* cell walls and inulin, it was decided to employ single point analysis of partial lysis and the Von Krogh conversion factors to determine residual $C'H_{50}$ unit activity (25). It was further felt that some relationship should be established between the amount of complement found present in VBD controls by titration and the amount established by single point analysis. This was accomplished by performing single point analysis of VBD controls. In all cases there was a difference in the amount of complement determined in these controls by titration from that established by calculation using the Von Krogh conversion factors. This difference was expressed as a percent of the number of units found by titration and ranged from 1.7% to 7.3%.

Based on the earlier tests that indicated that the anticomplementary activity of *F. polymorphum* might be localized in cell wall fragments (C. E. Hawley and W. A. Falkler, Jr. In preparation), it was decided to select the cytoplasm free cell wall preparations as the standard anticomplementary agent. It also seemed logical that a study of the anticomplementary activity by the cell walls of *F. polymorphum* would have more relevance to the pathogenesis of clinical disease.

Bladen *et al.* (3) were able to progressively add LPS to a fixed amount of GPC' and show that the reaction of LPS with GPC' was stoichoimetric in nature. They also showed with a sigmoid curve that between 100 and 200 µg of the LPS preparation would achieve maximum consumption of the GP $C'H_{50}$ unit activity present. The same apparent relationship was reported by Pillemer *et al.* (35) between zymosan and human C'3. It therefore seemed necessary to determine if this sigmoidal relationship that had been observed between the concentration of anticomplementary agent and the amount of complement consumed could be produced with *F. polymorphum* cell walls. A titration of cell walls was performed using dry weights from 31.25 to 1,000 µg with a fixed amount of GPC'. The results of the titration (Figure 1) showed that maximum anticomplementary activity was recorded at 500 µg. From the data presented, it was decided to use 500 µg of cell walls in future tests where maximum consumption of $C'H_{50}$ unit activity would be required. In addition, the titration curve (Figure 1) was used to help select dry weights of FP cell walls where sub-optimal consumption would be advantageous to the assay.

It was also suggested that the binding of limiting factors in the 1:10 dilution of GPC' was saturated by 500 µg of the cell wall preparation. Some information about the nature of limiting factor(s) was provided in the tests where heat treated GPC' was added to the complement consumption assay without producing

any increase in complement consumption (Table 1). Based upon the reports in the literature which were concerned with the characterization of serum factors in the properdin system, we know that there are at least two heat labile (50 C for 30 min) factors (35) required for the inactivation of C'3 by zymosan. One of these, Factor B or C3PA, has the same heat lability (50 C, 30 min) and electrophoretic characteristics (β pseudoglobulin) as C'2, yet shows no functional relationship to C'2, (13, 14). It would seem that the results here are consistent with these earlier reports, and in addition, there are a fixed number of sites in a given volume of GPC' that react directly or indirectly with activators of the alternate pathway. The addition of agents in excess of these sites has no effect on increasing the consumption of total complement.

FP cell walls were also titrated with GPC' for one hour at 0 C. In contrast to earlier reports by Bladen *et al.* (3) and Pillemer *et al.* (34,35) where there was no measurable consumption of total complement or C'3 from 3 to 48 h at 0 C, here there was a low level of $C'H_{50}$ unit consumption that ranged from 0% for 62.5 µg to 20.1% for 1,000 µg of the FP cell walls. The discrepancy between the results reported here and the earlier reports could be explained on the basis of differences in the agents used, their concentration, and the design of the test systems employed. On the basis of the titration curves in Figure 2, it appears that the same reactions that functioned optimally at 37 C also functioned suboptimally at 0 C and were responsible for the observed reduction in total complement. Since it was also observed that the addition of 2,000 µg of cell walls to the complement consumption assay produced apparent maximum consumption of GPC' at 0 C, a functional parallel may be drawn between the kinetics of reactions that occur at both 37 C and 0 C. The results of tests with GPC' and the cell walls at either temperature suggest that there is a critical number of reactive sites in GPC' that must be bound by the cell

walls in order to ensure optimal complement consumption. The binding of these sites is apparently influenced by the degree of molecular interaction, which is temperature and concentration dependent.

It was determined here that the limiting factor in GPC' which restricted the amount of consumption by a given concentration of cell walls was labile at 56 C for 30 min. This was shown by investigating the consumption of normal GPC' by a sub-optimal concentration of cell walls in the presence of two and four fold additions of heat treated (56 C for 30 min) or normal GPC'. Heat treatment was employed to totally inactivate C1 and C2 and to partially inactivate C3 and C4 (25). The same treatment would also inactivate the heat labile non-immunoglobulin serum factors essential to the alternate pathway (13, 14, 35). The use of sub-optimal concentrations of FP cell walls (125 µg) was therefore considered critical in order that sufficient total complement be available in the system to detect augmentation of consumption by heat stable antibodies in the added heat treated GPC'. The presence of unconsumed total complement activity in the test with 125 µg of cell walls was suggested by the higher levels of consumption shown with 500 µg of cell walls. This test showed (Table 1) that two and four fold additions of heat treated GPC' to the test did not augment its consumption suggesting that GPC' did not contain antibody that could have reacted with cell walls in the complement consumption assay. However, the two and four fold additions of normal GPC' contributed to two and four fold increases in the number of $C'H_{50}$ units of complement activity consumed.

It was also of interest that while the number of units consumed increased with the addition of normal GPC', the percent consumption of units in the test remained essentially unchanged. Again, the amount of consumption depended upon the relative proportions of cell walls and heat labile factor(s) in GPC'. If the amount of limiting factor was doubled with the same concentration of cell

walls, the consumption of complement predictably doubled as proportionally more cell wall active sites are involved. But, in all instances the ratio of complement consumed to total complement present was the same and was dependent upon the amount of cell walls present.

While the alternate pathway of complement consumption by laboratory preparations of *F. polymorphum* had been suggested by the preceding discussion, the confirmation of strict alternate pathway activity would require the use of C4D (5, 7, 40). Prior to its use in the complement consumption assay here, the hemolytic deficiency in the C4D was first confirmed by the inability of C4D to lyse EA cells in a microtiter test. The same EA could be lysed at the same time in control tests with normal GPC'. The restoration of hemolytic activity in C4D was demonstrated through the lysis of EAC'1,4 cells.

The results shown in Table 2 revealed significant anticomplementary activity by both cell walls and inulin at 500 µg each in the complement consumption assay using C4D. It was further shown here that EA cells could not lyse in the presence of the same dilutions of C4D that did lyse EAC'1,4. This indicated that the classical complement pathway could not have functioned during the incubation of anticomplementary agents with C4D. A alternate complement pathway activation by cell walls and inulin without the participation of C4 was therefore confirmed. Progressive increases in C4D consumption were recorded with increasing concentrations of cell walls in the assay which suggested C4D consumption kinetics similar to GPC'.

On the basis of the findings reported here, the potential for activation of the alternate complement pathway by factors in the cell walls of *F. polymorphum* was supported. The exclusive activation of the alternate pathway, without the influence of early complement components, was confirmed with C4D. The potential for similar anticomplementary activity *in situ* and the generation

of phlogistic active complement components by *Fusobacterium* species as part of the immunopathology of periodontal diseases may occur, but further investigations demonstrating the production of such factors will be required.

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The Materials and Methods presented in this manuscript are basic to current mission oriented research activities at USAIDR, Washington, D.C.

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Decrease in Total C'H50 Units
0
2
4
6
8
10
12
14
VBD**
1000 µg
900 µg
800 µg
700 µg
600 µg
500 µg
400 µg
300 µg
200 µg
100 µg
Dry Weight FP Cell Walls in Test
Percent Decrease in Total GPC' C'H50 Unit Activity(37 C for 1H)
0
10
20
30
40
50
60
70

Figure 1.

Figure 1. This figure shows the anticomplementary effect of different concentrations of cell walls on a fixed amount of GPC' (28.14 $C'H_{50}$ units). The parallel curves represent the total units consumed (Δ) and the percent units consumed (●) by 31.25 µg, 62.5 µg, 125 µg, 250 µg, 500 µg, and 1,000 µg dry weight of the FP cell wall preparation.

Percent Decrease in Total C'H50 Unit Activity (1H)
0
10
20
30
40
50
60
70
Dry Weight FP Cell Walls in Test
100 µg
200 µg
300 µg
400 µg
500 µg
600 µg
700 µg
800 µg
900 µg
1000 µg
2000 µg
VBD**
37C
0 C

Figure 2.

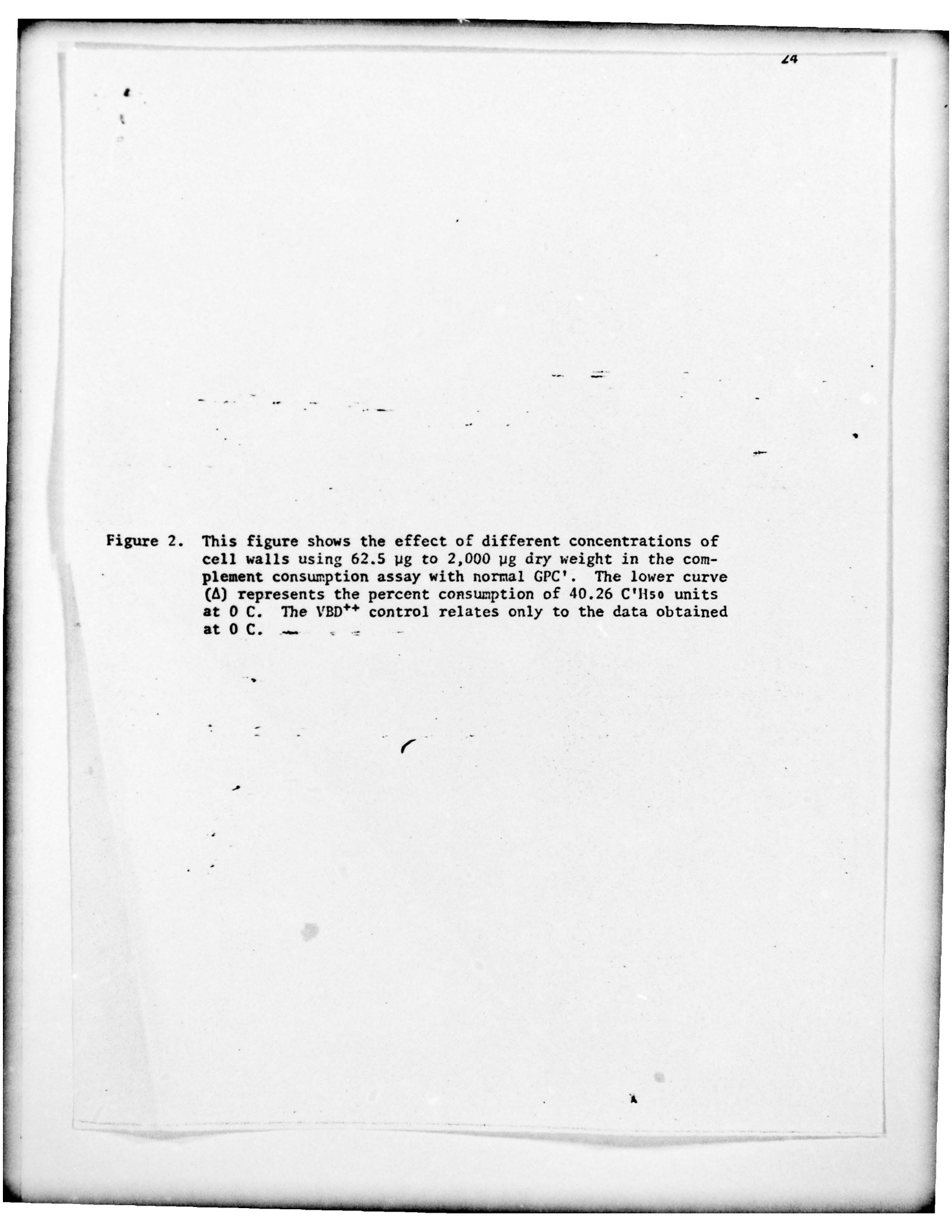

Figure 2. This figure shows the effect of different concentrations of cell walls using 62.5 µg to 2,000 µg dry weight in the complement consumption assay with normal GPC'. The lower curve (Δ) represents the percent consumption of 40.26 C'H$_{50}$ units at 0 C. The VBD^{++} control relates only to the data obtained at 0 C.